The T Cell Death Knell: Immune-mediated Tumor Death in Renal Cell Carcinoma


Departments of Immunology [R. G. U., V. K., C. T., L. M., R. B., J. H. F.], Urology [R. G. U., A. C. N., R. B., J. H. F.], and Experimental Therapeutics [V. K., C. T., L. M., R. B., J. H. F.], The Cleveland Clinic Foundation, Cleveland, Ohio 44195; Department of Medicine, Evanston Hospital, Northwestern University, Evanston, Illinois 60208 [C. J. F.]; Department of Urology, Division of Urologic Oncology, The New York Hospital Cornell University Medical Center, New York, New York 60208 [N. H. B.]

ABSTRACT

The antitumor effect of T cells is executed either through CD95 or Perforin (PFN)/Granzyme B (GrB) pathways. Induction of apoptosis by either mode requires activation of caspase family members. However, recent studies have suggested that cell death can proceed in the absence of caspase induction and apoptotic events. We investigated the contribution of CD95 and PFN/GrB-mediated cytotoxicity to apoptotic and necrotic mechanisms of cell death in human renal cell carcinoma. Although freshly isolated and cultured tumors expressed CD95 on their surface, they were resistant to CD95-mediated apoptosis. CD95 resistance increased with decreased levels of FADD protein and diminished caspase-3-like activity. In contrast, we demonstrated that tumor cell death mediated by PFN/GrB can be achieved in the absence of functional caspase activity and is accompanied by a dramatic accumulation of nonapoptotic necrotic cells.

INTRODUCTION

Two distinct molecular mechanisms have evolved as effectors of CTL-mediated cell death: one based on ligation of the CD95 receptor on target cells, and the other through directed exocytosis of GrB and PFN. Both are capable of initiating the apoptotic program in susceptible target cells. Engagement of CD95 by its ligand triggers a cascade of events, including aggregation of its intracellular death domains (2) and proteolytic activation of downstream caspases (3). Granule-induced CTL apoptosis also involves caspase-dependent mechanisms. Transfer of the effector serine proteases, granzymes from the CTL to the target cell is facilitated by the pore-forming protein PFN (4). One member of the granzyme family, GrB, exhibits specificity for aspartate residues (1) and is therefore capable of processing procaspases to form active heterodimeric enzymes, thereby triggering the caspase cascade and initiating the death program (5). Tumors may overcome their sensitivity to apoptotic stimuli by selective defects in the intracellular signaling proteins and proteases central to apoptotic pathways (6). These defects may provide a selective advantage for transformed cells, thereby rendering them resistant to various forms of antitumor chemotherapies and immunotherapies dependent on the induction of apoptosis in tumor targets (7). However, although defects in caspases and other elements of the apoptotic program can prevent programmed cell death, they do not always prevent irreversible loss of cellular function.

We investigated the susceptibility of RCC cells to apoptotic and necrotic modes of cell death initiated by the two primary CTL effector mechanisms, CD95 and PFN/GrB. We demonstrate that despite normal surface expression of CD95, tumor cells were resistant to apoptosis induced by ligation of the receptor. Tumor cells were also resistant to apoptosis induced by PFN/GrB, which failed to initiate any caspase-like activity. However, despite these endogenous defects, the addition of PFN and GrB to caspase-deficient RCC induced nonapoptotic necrotic cell death. These data demonstrate that CTL may overcome tumor resistance to apoptotic modes of cell death via GrB-mediated cellular necrosis, supporting the development of immunotherapeutic strategies for the treatment of RCC.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Three established and previously characterized RCC lines (7, 26B, and 48) were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), 1-glutamine (2 mM), gentamicin (50 mg/l), sodium pyruvate (1 mM), and non-essential amino acids (0.1 mM). Cultures were maintained at 37°C with 5% CO₂. Jurkat cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in supplemented medium in an identical fashion.

Antibodies and Reagents. Antibodies used in immunocytochemistry for CD95 were obtained as FITC conjugates (anti-CD95; Becton Dickinson, San Jose, CA). Antibody used in Western blotting for FADD was purchased from Transduction Labs (Lexington, KY); antiactin antibody was obtained from Sigma Chemical Co. (St. Louis, MO). Secondary horse radish peroxidase-conjugated sheep antimouse antibodies were pur-
chased from Amersham (Arlington Heights, IL). Transactivating anti-CD95 antibody, CD95L, and its potentiator were obtained from Upstate Biotechnology (Lake Placid, NY). The caspase-3 (DEVD-AMC) and the caspase-8 (IETD-AMC) substrates were purchased from Calbiochem (San Diego, CA).

**Immunocytometry and Western Blot Analysis.** Cells were stained with anti-CD95 antibody and analyzed by flow cytometry. Live gating of the forward and orthogonal scatter channels was used to exclude debris and to selectively acquire lymphocyte or epithelial cell events. Individual fluorescence data were determined through the use of acquisition and quadrant analysis software (CELLQUEST; Becton Dickinson). For all experiments, matched isotype controls were obtained in an identical manner. Western blotting was performed as described previously (6).

**Induction and Measurement of Apoptosis and Necrosis.** Tumor cells were cultured in supplemented medium and grown to confluence. Transactivating anti-CD95 antibody or the soluble CD95L and its potentiator were added to RCC lines and Jurkat controls as indicated. Human PFN and GrB were purified as described previously (8). The protocol for induction of cell death by these reagents has been outlined elsewhere (4). Sublytic doses of PFN were used at 200–350 units/ml, which were

![Figure 1](image-url)

**Fig. 1** Human RCC lines are resistant to CD95-mediated cell death. Three established tumor cell lines were cultured in the absence (Medium) or presence of transactivating monoclonal anti-CD95 antibody (250 ng/ml; αCD95 Ab) or soluble CD95L (200 ng/ml) and its potentiator (1.5 μg/ml) for 18 h. Jurkat cells treated with anti-CD95 antibody (50 ng/ml; αCD95 Ab) or soluble CD95L (50 ng/ml) and its potentiator (1.5 μg/ml) served as a positive control. A, apoptotic DNA fragmentation was determined by TUNEL as described in “Materials and Methods.” The X-axis represents DNA content; the Y-axis represents fluorescence intensity. B, aliquots of the same cells were used to measure the percentage of PI-positive cells. The Y-axis represents relative fluorescence intensity; the X-axis represents cell number.
defined as <10% PI staining in cells treated with PFN alone. GrB was used at 2.5 μg/ml. Cells were treated for 6 h prior to TUNEL assay and PI staining.

The APO-BRDU kit (Phoenix Flow Systems Inc., San Diego, CA) was used to detect DNA fragmentation according to the protocol provided. Necrotic cells were measured by FACS analysis of PI-stained nuclei as previously described (9). Assessment of Functional Caspase Activity. Caspase-3 and -8 activity was measured using the fluorometric tetrapeptide substrates, DEVD-AMC and IETD-AMC, respectively. The assays were performed in 96-well plates by incubating 20 μg of cell lysates with 180 μl of reaction buffer [100 mmol/L HEPES (pH 7.5), 20% (v/v) glycerol, 5 mmol/L DTT, and 0.5 mmol/L EDTA] containing 50 μM DEVD-AMC or IETD-AMC. AMC release was monitored after 1 h incubation at 37°C on a microplate fluorometer with excitation and emission wavelengths of 380 and 460 nm, respectively.

RESULTS AND DISCUSSION

Human RCC Cell Lines Express CD95 Receptor but Are Resistant to CD95-mediated Cell Death. The CD95 receptor-ligand system plays a crucial role in various physiological and pathological forms of cell death and is one of the two effector pathways that CTLs use to rapidly kill targets. Expression of CD95L on the surface of CTLs cross-links CD95 receptor on target cells, thereby signaling aggregation of its intracellular death domain and formation of the DISC (3), which initiates the apoptotic program.

Here we investigated the sensitivity of RCC cell lines to CD95-mediated cell death. Using three well-characterized RCC cell lines, we demonstrated complete resistance to transactivating anti-CD95 monoclonal antibody as well as soluble CD95L, although both agents were able to induce a high level of apoptosis in a control Jurkat cell line (Fig. 1A). To further document that CD95 activation has no effect on viability of RCC cell lines, we used PI staining and FACS analysis. In viable cells, cell membranes are impermeable to PI. In contrast, only dead cells whose plasma membranes have been disrupted will incorporate PI. As expected, apoptosis-resistant RCC cell lines also failed to incorporate PI in response to CD95 activation, confirming their viability despite delivery of a death signal (Fig. 1B).

To investigate the mechanisms of resistance to CD95-mediated apoptosis, we next evaluated expression of CD95 on the tumor cell lines. All tested RCC cell lines expressed signif-
Significant levels of the receptor on the cell surface as measured by immunocytometry (Fig. 2A). These findings are consistent with those in other tumor models, including prostate (10), melanoma (11), and T-cell lymphoma (12), demonstrating that CD95 expression is a necessary but insufficient predictor of CD95 sensitivity in human malignancies. Furthermore, recent evidence suggests that drug-induced apoptosis in tumor cells is mediated in part by the CD95 receptor/ligand system (13, 14) and that tumor cell resistance to apoptotic signals mediated via death receptors correlates with resistance to apoptosis induced by antineoplastic agents (7). Strategies aimed at increasing CD95 expression on tumor cells to render them more sensitive to chemo- or immunotherapies are therefore unlikely to succeed.

Resistance to CD95-mediated Apoptosis Coincides with Reduced Expression of the Adaptor Protein FADD and Diminished Activation of Caspase-8. Activation of the proteolytic caspase cascade represents the effector component of the CD95 pathway. Ligation of the surface CD95 receptor and formation of the DISC involves activation of the initiator caspases. Coupling of the receptor signal occurs through the intracellular protein FADD (MORT-1), which binds through its COOH-terminal death domain to cross-linked CD95 receptors and recruits caspase-8 through its NH2-terminal death effector domain to the DISC (3, 15, 16). Activation of the procaspase by association with the CD95/DISC leads to subsequent down-stream activation of effector caspases and ultimately DNA fragmentation factor (DFF-45).

During the process of malignant transformation, multiple defects along the CD95 signaling complex may provide cells a selective advantage that renders them insensitive to CD95-mediated death signals despite normal expression of the CD95 receptor. Although we have previously demonstrated that RCC lines exhibit variable decreased expression of several members of the caspase family, recent evidence suggests that Fas-associated protein with death domain can kill cells via two pathways, one mediated by caspases and another that does not involve them (17). These data suggest that variable caspase deficiencies do not ultimately result in complete resistance to CD95-mediated cell death. Previous work has demonstrated that tumor cells expressing a splice variant coding for a truncated Fas molecule that lacks the intracytoplasmic signaling domain are resistant to apoptosis (12). We therefore evaluated expression of the most upstream protein involved in CD95 signaling, coupling of the intracellular receptor complex to the caspase cascade by the adapter protein FADD. As demonstrated in Fig. 2B, expression of this death domain is markedly reduced in RCC lines and may contribute to CD95 insensitivity at the most apical level. As expected, treatment with soluble CD95L failed to induce...
caspase-3- and caspase-8-like activity in the RCC cell lines, whereas such activity was easily detectable in Jurkat cells (Fig. 3C).

**PFN/GrB Induces Necrotic Cell Death in RCC Cell Lines with Defective Apoptotic Machinery.** CTLs use a second major effector pathway requiring exocytosis of preformed granules that act as lytic effectors and initiate target cell death. This mechanism requires the pore-forming protein PFN, which inserts into the target cell membrane and facilitates entry and release from endosomal compartments of the serine protease GrB, which is capable of processing caspases and initiating apoptosis (4). Interestingly, whereas nuclear apoptosis induced by GrB depends entirely on activation of various caspses, Granzyme A-induced nuclear condensation and DNA damage are independent of caspase activation or caspase substrate cleavage (18). Because both the PFN/GrB and the CD95 receptor/ligand systems induce apoptotic changes in target nuclei, it has been assumed that they share downstream pathways common to the apoptotic program (19). To study whether RCC lines with defective apoptotic machinery are susceptible to PFN/GrB-mediated cell death, we treated tumor cells with these agents and examined them for evidence of DNA fragmentation by TUNEL assay (Fig. 3A). Despite good induction of apoptosis in Jurkat T-cell controls, there was little evidence for apoptotic cell death in RCC lines treated in this manner. Resistance to apoptosis under these conditions correlated with failure to induce caspase-3-like activity in the RCC cell lines evaluated, whereas caspase-3-like activity was easily detectable in Jurkat cells (Fig. 3B).

It has previously been established that although apoptosis induced by GrB depends on activation of members of the caspase family, target cell lysis may occur via a caspase-independent pathway (19). To further investigate whether RCC lines undergo nonapoptotic cell death in response to PFN/GrB, we used PI staining. As demonstrated in Fig. 4, in contrast to the negligible effect on the induction of apoptosis, treatment with these agents resulted in significant accumulation of PI-positive cells in all RCC lines evaluated. Renal carcinoma cells died only when they were exposed to both PFN and GrB simultaneously, whereas cells exposed to PFN or GrB alone maintained viability (Fig. 4). Whether PFN alone is capable of lysing target cells in vivo or serves only as a transport molecule for GrB delivery is still unclear. These data support the finding that PFN/GrB-mediated nonapoptotic cell death can proceed in the absence of functional caspase activity in tumor cells, whereas in the caspase-competent Jurkat cells, these agents initiate apoptotic cell death. Therefore, the presence or absence of functional caspase activity determines the mode of cell death in response to this CTL-mediated pathway rather than determining whether death is the ultimate consequence.

These data demonstrate that despite acquired defects in the apoptotic machinery that are selectively advantageous to renal carcinoma cells and may render them insensitive to cytotoxic agents, immune effector cells may eliminate tumors resistant to apoptosis by initiating nonapoptotic cell death via PFN/GrB. This may have significant implications for the further development of new immunotherapeutic strategies aimed at overcoming resistance of tumor cells to conventional therapies. Furthermore, it is essential not only to monitor nonapoptotic death events, but also to develop therapeutic strategies targeting this alternative form of tumor cell death.

**ACKNOWLEDGMENTS**

We thank Amy Raber for expert technical assistance.
REFERENCES


Clinical Cancer Research

The T Cell Death Knell: Immune-mediated Tumor Death in Renal Cell Carcinoma


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/10/3276

Cited articles
This article cites 19 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/10/3276.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/7/10/3276.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.